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Evaluation of Protein Quality in Microbound Starter Diets Made with Decapsulated Cysts of *Artemia* and Fishmeal for Fish Larvae

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Abstract.—The protein quality of carboxymethylcellulose microbound diets (MBDs) made with decapsulated cysts of *Artemia* and/or fishmeal as protein sources was used as an indicator of their suitability as starter feed for fish larvae. Studies on the proximate, fatty acid and amino acid composition, *in vitro* protein digestibility, diet solubility, and protein structure were combined with an *in vivo* feeding experiment with African catfish *Clarias gariepinus* larvae to evaluate the protein quality of the MBDs and a commercial diet. The growth of catfish larvae was higher when fed *Artemia*-based MBDs than with fishmeal-based MBDs, despite the higher protein and amino acid content of the latter. The *in vitro* protein digestibility was high for all the MBDs in comparison to a commercial diet. Differences were found in the protein molecular weight among the diets. Most of the proteins in the fishmeal-based diets had low molecular weight in the range between 7.4 and 49.2 kDa. The *Artemia*-based MBDs had larger protein fractions between 29.4 and 82 kDa. Decapsulated cysts improved the utilization of the MBDs when used in combination with fishmeal. Besides the effect of chemical attractants, the explanation for the positive effect of *Artemia* has yet to be elucidated. However, attention should be given to interactions between nutrients (e.g., protein-lipid) in live food, which might have an effect on the functional properties of food proteins.

The first feeding of many fish larvae relies on the use of live food organisms (e.g., rotifers and *Artemia*). The numerous attempts to replace live food by artificial starter diets had limited success so far, i.e., the highest growth in larval fish is still obtained with live food (Jones et al. 1993; Watanabe and Kiron 1994). One of the hypotheses that has been proposed to explain this suggests the importance of the high content of free amino acids (FAA) in live food organisms (Dabrowski and Rusiecki 1983; Fyhn et al. 1993). The supply of FAA and low molecular weight peptides in starter diets may facilitate their intestinal absorption by fish larvae (Fyhn 1989; Walford and Lam 1993; Rønnestad et al. 1999). Similarly, it can be suggested that the structure of the proteins in live food, which might be more easily digested by the fish larvae (García-Ortega et al. 2000a), might differ from that of the proteins used in the manufacturing of artificial diets. Additional support for this hypothesis is found in the different growth results when fish larvae are fed with either decapsulated cysts or nauplii of *Artemia*. On an individual weight basis, the decapsulated cysts and nauplii of *Artemia* have similar biochemical composition in all the major nutrients (García-Ortega et al. 1998). Thus, with regard to the amount of nutrients, there is no difference in feeding *Artemia* cysts or nauplii to fish larvae. However, in some species higher fish growth is achieved with nauplii (Van-

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haecke et al. 1990). Protein is the major component of the dry matter in *Artemia*. Because the interaction of proteins with water has an effect on the functional properties of the protein (Damodaran 1997), the protein structure might differ between cysts and nauplii due to the high water content in the latter.

The aim of the present study was to evaluate the quality of different protein sources in microbound diets (MBDs) for fish larvae and to relate the differences in performance to the protein structure. In addition, the suitability of decapsulated cysts of *Artemia* as a protein source in starter diets for fish larvae was evaluated. MBDs were selected as experimental diets because they were used successfully in the larval rearing of African catfish (Pector et al. 1994), red sea bream, and Japanese flounder (Kanazawa et al. 1989). Decapsulated cysts of *Artemia* were used as a protein source in experimental MBDs for fish larvae because cysts have similar nutritional composition as nauplii (the control diet in the present study), they are well accepted by the fish larvae, and have high digestibility coefficients above 75% total protein (Pector et al. 1994; García-Ortega et al. 1998).

The quality of protein in fish feeds has been previously studied by diverse *in vitro* techniques (Grabner 1985; Dimes and Haard 1994) in combination with electrophoretic methods (Bassompierre et al. 1998; Alarcón et al. 1999). *In vitro* techniques permit simple, fast and reproducible methods for the evaluation of nutrient digestibility (Boisen and Eggum 1991). However, only a few studies utilized *in vitro* techniques for the determination of protein digestibility in larval fish feeds (Ozkizilcik and Chu 1996; Alarcón et al. 1999; García-Ortega et al. 2000a). None of the previous studies used *in vivo* growth experiments to test the quality of their larval diets. For a more complete picture of the protein quality in fish feeds, *in vivo* growth trials can be combined with studies on amino acid composition, *in vitro* digestibility, diet solubility and studies on protein structure. This combined approach was followed in our investigations.

Materials and Methods

The present study was composed of two parts. In the first part a feeding experiment was done to test the growth response of African catfish *Clarias gariepinus* larvae to four experimental MBDs made of decapsulated cysts of *Artemia* or fishmeal as protein sources, one commercial starter feed and *Artemia* nauplii. In the second part, a set of analyses to evaluate the quality of protein in the tested diets was done. The criteria to determine protein quality in the diets were proximate and amino acid composition, *in vitro* digestibility, and analysis of protein structure or molecular weight by electrophoresis.

Diet Preparation

Cysts of *Artemia* from Great Salt Lake, USA (EG type, INVE Aquaculture NV, Belgium) were decapsulated with a hypochlorite solution (Sorgeloos et al. 1986), dehydrated in brine (>330 g NaCl/L) during 24 h and stored in fresh brine at 4 C for one day until further processing. The quality of this type of cysts has been previously tested (García-Ortega et al. 1998). Decapsulated cysts of *Artemia* and fishmeal were used alone or in combination as protein sources in the MBDs. Because cysts and fishmeal have other nutritional components besides protein, the formulation of the experimental MBDs was done to obtain a similar protein composition among the different diets (Table 1). Each MBD was prepared by mixing all the ingredients with carboxymethyl-cellulose (CMC) (10% dw) and water. An advantage of the use of CMC as a binder is that the application of heat during diet manufacturing is avoided. This facilitates the use of *Artemia* as a dietary ingredient because processing food protein with heat might produce negative effects on the protein quality (Boye et al. 1997). After thorough mixing of the ingredients, the

TABLE 1. *Formulation and proximate composition (g/100 g dw) of experimental microbound diets made with decapsulated cysts of* Artemia *(MBD 1 and MBD 2), a combination of cysts and fishmeal (MBD 3), and fishmeal (MBD 4) as protein sources, and proximate composition of a commercial diet and instar I nauplii of* Artemia.

	Diet					
	MBD 1	MBD 2	MBD 3[a]	MBD 4	Com.	Nauplii
Formulation						
Artemia cysts[b]	90		47.2			
Artemia cysts heated at 80 C		90				
Fishmeal[c]			34	74.2		
Fish oil[c]			6.3	13.3		
Mineral and vitamin premix[c]			2.5	2.5		
CMC[d]	10	10	10	10		
Proximate composition						
Protein	46.4	52.6	52.7	56.4	60.6	60.6
Lipid	14.4	15.8	18.3	23.2	14.3	18.3
Ash	9.3	5.1	9.4	10.4	12.3	2.2
Dry matter	91.6	94.1	93.9	96.1	96.4	17.4

[a] Total protein in MBD 3 was approximately 48% from cysts (47 g protein in 100 g dw of cysts) and 52% from fishmeal (71 g protein in 100 g dw of fishmeal).
[b] INVE Aquaculture NV, Baasrode, Belgium.
[c] Low temperature fishmeal, Provimi, BV, Rotterdam, The Netherlands.
[d] Carboxymethylcellulose Sodium salt, Fluka Chemie, Buchs, Switzerland.

paste was milled and dried at 40 C, then ground and sieved to a particle size of 300–500 μm. In MBDs 1 and 2, decapsulated cysts of *Artemia* were included as the only feed ingredient with CMC. Previous to preparation of MBD 2, the decapsulated cysts were hydrated and then submitted to a heat treatment in water at 80 C for 5 min to reduce the protein quality (García-Ortega et al. 2000b). For MBD 3, approximately 48% of the protein originated from decapsulated cysts and 52% from fishmeal. In MBD 4 all protein originated from fishmeal.

Feeding Experiment

Eggs and larvae of African catfish were obtained by artificial reproduction of controlled broodstock. Two days after hatching, the larvae were counted and transferred to a recirculation system with 17-L aquaria at a density of 66 larvae/L. Fish larvae rearing was done at a constant temperature of 28 C and a light regime from 0800 h to 2000 h. Four experimental MBDs, one commercial starter feed (Com.; Nippai, Japan), and live *Artemia* nauplii as control diet were all tested in triplicate. Two days after hatching, once the yolk sac was completely absorbed, exogenous feeding was initiated. The amount of food offered was calculated everyday taking into account the estimation for maximum growth in *C. gariepinus* larvae according to Verreth and Den Bieman (1987). Feed was administered five times a day (every 4 h) from 0800 h to 2300 h. The fish weight was determined daily in all aquaria by taking a sample of 20 fish per aquarium. The fish were siphoned out of the aquaria and kept in a net where excess water was removed with tissue paper before weighing in aluminum foil cups. The mean initial wet weight of the fish larvae was 2.7 (±0.1) mg. The total duration of the feeding experiment was nine days.

Proximate Composition and Diet Solubility

The protein, lipid, ash, individual amino acid and fatty acids compositions of the four MBDs, the commercial feed and instar I nauplii of *Artemia* were determined as

previously reported by García-Ortega et al. (1998). The dry matter of diets and fish larvae were estimated by drying the samples in an oven at 60 C for 24 h. The solubility of the MBDs was determined by exposing approximately 0.25 g of the feed to 50 mL distilled water in a rotating tube for 60 min. The dissolved matter was separated by centrifugation at 2000 × *g* for 3 min, and the dry weight was then determined. The protein content of the insoluble part after the solubility test also was determined.

Protein Digestibility in vitro

A multi-enzyme system to simulate the conditions in which food digestion occurs in fish larvae without a functional stomach (García-Ortega et al. 2000a) was used to estimate the *in vitro* protein digestibility of larval fish diets. MBD samples (±400 mg) and instar I nauplii of *Artemia* were incubated at 30 C and pH 8 for 4 h in a solution of purified enzymes. The enzyme mix consisted of a solution of 0.1 mL cod *Gadus morhua* trypsin (T-9906, Sigma-Aldrich, Zwijndrecht, The Netherlands), 1 mL chymotrypsin from bovine pancreas (C-4129, Sigma), 0.1 mL leucine-aminopeptidase from porcine kidney (L-1503, Sigma), 1 mL α-amylase (Termamyl 120L, Novo Nordisk, Denmark), 40 mg lipase from wheat germ (L-3001, Sigma), and 80 g bile salts (B-8756, Sigma) dissolved in a solution of Na_2HPO_4 and KH_2PO_4. To stabilize the enzymes in the solution, 1 mL $CaCl_2$ and 0.1 mL $MgCl_2$ with 0.1 mL $(NH_4)_2SO_4$ were added. After incubation in the multi-enzyme solution, the digested fraction was vacuum-filtrated and the undigested protein fraction was determined by the Kjeldahl method. The protein digestibility *in vitro* was estimated from the original nitrogen content of the MBD and the undigested fraction after incubation.

SDS-PAGE and Molecular Weight Determination

Protein in the diet samples and instar I nauplii of *Artemia* was extracted with 6 M urea in a phosphate buffer saline (PBS, 0.9%) at pH 7.3 and subsequently ground for 2 min. The mixture was centrifuged for 3 min at 2000 × *g*, and the supernatant collected and diluted with distilled water (1:1). The sample was heated at 100 C for 3 min in the presence of 2-mercaptoethanol sodium dodecyl sulfate (SDS) (1:3) to dissociate the proteins into their component polypeptides. Separation of proteins in the diets was done by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli (1970). A running gel containing 10% polyacrylamide and a stacking gel containing 4% polyacrylamide were used. The protein standards (broad range 161–0318, Bio-Rad Laboratories, California, USA) were myosin (200 kiloDaltons), β-galactosidase (113 kDa), bovine serum albumin (82 kDa), ovalbumin (49.2 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (29.4 kDa), lysozyme (20.9 kDa) and aprotinin (7.4 kDa). The protein in samples and the protein standards were run in a Bio-Rad Mini-Protean II cell in two programs, with 80 V applied for 40 min and 120 V for 90 min, respectively. The gel was stained with Coomassie Brilliant Blue (R-250, Sigma) in a solution of methanol, acetic acid, and water (5:1:5). Destaining was done with a solution of methanol, acetic acid, and water (3:1:10). The molecular weight estimation of the proteins in the gel was done with a laser densitometer (UltroScan XL, Pharmacia LKB Biotechnology, Uppsala, Sweden) and the Gel Scan XL software (Pharmacia LKB Biotechnology). After scanning the gel, the equation of Ferguson (1964) was used to determine the relative mobility (R_f) of the polypeptides in the acrylamide. By plotting the logarithmic R_f values from the standard proteins against the known acrylamide concentration, straight lines and slopes of K_R (retardation coefficient) were obtained. The K_R values were plotted against the molecular weight of the standard proteins. A linear relationship was obtained which was used to estimate the molecular weight of

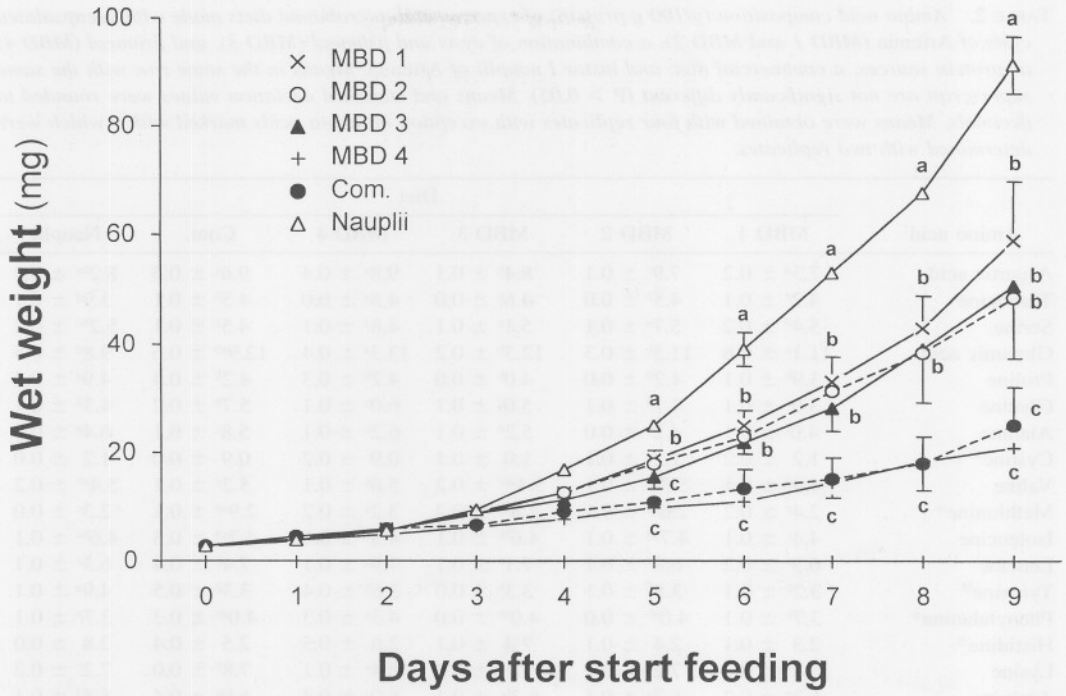

FIGURE 1. *Daily growth of* Clarias gariepinus *larvae fed four experimental microbound diets (MBD), one commercial diet, and nauplii of* Artemia *during the first days of exogenous feeding. Means in the same day with different letter are significantly different* ($P < 0.05$).

unknown proteins. The distribution of the protein polypeptides from the diets among the molecular weight of each standard protein is expressed as percentage of the relative area found for each band in the gel.

Data Analysis

The weight data of three replicate aquaria per treatment were used for statistical analysis. The individual amino acid content was determined with four samples per diet, with exception of cystine, methionine, tyrosine, histidine and phenylalanine, which were determined with two samples. Fatty acids and diet solubility were determined in triplicate for each diet; *in vitro* protein digestibility was determined in duplicate. All data were tested for normal distribution and homogeneity of variance before ANOVA was done (Sokal and Rohlf 1995). To detect diet effects ($P < 0.05$) on fish weight, a one-way ANOVA was performed. It was also used to detect differences in the diets amino acid and fatty acid contents, in the diets solubility, and on their protein digestibility. When significant effects were found, the means were compared with Duncan multiple range test and least squares means for equal and unequal sample size, respectively. Statistical analyses were performed with SAS 6.12 (SAS Institute Inc., North Carolina, USA).

Results

Fish Growth

For the first 3 d after the start of exogenous feeding the catfish larvae showed similar growth in all the dietary treatments (Fig. 1). From that day onwards, the larval growth differed significantly ($P < 0.05$) among the different diet treatments. Three groups of treatments can be recognized according to larval growth (Fig. 1). The highest fish growth (90.9 mg) was achieved with live *Artemia* nauplii. The larvae fed

TABLE 2. *Amino acid composition (g/100 g protein) of experimental microbound diets made with decapsulated cysts of* Artemia *(MBD 1 and MBD 2), a combination of cysts and fishmeal (MBD 3), and fishmeal (MBD 4) as protein sources, a commercial diet, and instar I nauplii of* Artemia. *Means in the same row with the same superscript are not significantly different (P > 0.05). Means and standard deviation values were rounded to decimals. Means were obtained with four replicates with exception of amino acids marked with * which were determined with two replicates.*

	Diet					
Amino acid	MBD 1	MBD 2	MBD 3	MBD 4	Com.	Nauplii
Aspartic acid	$7.3^{d} \pm 0.2$	$7.9^{c} \pm 0.1$	$8.4^{b} \pm 0.1$	$9.8^{a} \pm 0.4$	$9.6^{a} \pm 0.5$	$8.2^{bc} \pm 0.2$
Threonine	$4.2^{c} \pm 0.1$	$4.5^{b} \pm 0.0$	$4.6^{b} \pm 0.0$	$4.8^{a} \pm 0.0$	$4.5^{b} \pm 0.1$	$3.9^{d} \pm 0.1$
Serine	$5.4^{b} \pm 0.2$	$5.7^{a} \pm 0.1$	$5.1^{c} \pm 0.1$	$4.8^{d} \pm 0.1$	$4.5^{e} \pm 0.1$	$5.2^{bc} \pm 0.2$
Glutamic acid	$11.1^{c} \pm 0.6$	$11.5^{c} \pm 0.3$	$12.3^{b} \pm 0.2$	$13.3^{a} \pm 0.4$	$12.9^{ab} \pm 0.6$	$9.8^{d} \pm 0.5$
Proline	$3.9^{b} \pm 0.1$	$4.2^{b} \pm 0.0$	$4.0^{b} \pm 0.0$	$4.2^{b} \pm 0.3$	$4.2^{b} \pm 0.4$	$4.9^{a} \pm 0.2$
Glycine	$3.9^{e} \pm 0.1$	$4.1^{d} \pm 0.1$	$5.0^{c} \pm 0.1$	$6.0^{a} \pm 0.1$	$5.7^{b} \pm 0.2$	$4.3^{d} \pm 0.1$
Alanine	$4.0^{f} \pm 0.1$	$4.2^{e} \pm 0.0$	$5.2^{d} \pm 0.1$	$6.2^{b} \pm 0.1$	$5.8^{c} \pm 0.1$	$6.4^{a} \pm 0.2$
Cystine*	1.2 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.0	1.2 ± 0.0
Valine	$4.9^{d} \pm 0.1$	$5.3^{bc} \pm 0.1$	$5.4^{abc} \pm 0.2$	$5.6^{a} \pm 0.1$	$5.2^{c} \pm 0.1$	$5.4^{ab} \pm 0.2$
Methionine*	$2.4^{c} \pm 0.2$	$2.6^{bc} \pm 0.1$	$2.8^{ab} \pm 0.2$	$3.2^{a} \pm 0.2$	$2.9^{ab} \pm 0.1$	$2.3^{c} \pm 0.0$
Isoleucine	$4.4^{c} \pm 0.1$	$4.7^{ab} \pm 0.1$	$4.6^{bc} \pm 0.1$	$4.8^{a} \pm 0.2$	$4.7^{ab} \pm 0.3$	$4.6^{bc} \pm 0.1$
Leucine	$6.3^{e} \pm 0.2$	$6.8^{d} \pm 0.1$	$7.1^{c} \pm 0.1$	$7.9^{a} \pm 0.1$	$7.4^{b} \pm 0.4$	$6.3^{e} \pm 0.1$
Tyrosine*	$3.2^{b} \pm 0.1$	$3.5^{b} \pm 0.1$	$3.3^{b} \pm 0.0$	$3.6^{b} \pm 0.4$	$3.3^{b} \pm 0.5$	$4.9^{a} \pm 0.1$
Phenylalanine*	$3.7^{b} \pm 0.1$	$4.0^{ab} \pm 0.0$	$4.0^{ab} \pm 0.0$	$4.3^{a} \pm 0.3$	$4.0^{ab} \pm 0.3$	$3.7^{b} \pm 0.1$
Histidine*	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.6 ± 0.5	2.5 ± 0.4	2.8 ± 0.0
Lysine	$6.8^{d} \pm 0.2$	$7.2^{c} \pm 0.1$	$7.7^{b} \pm 0.0$	$8.4^{a} \pm 0.1$	$7.8^{b} \pm 0.0$	$7.2^{c} \pm 0.2$
Arginine	$6.3^{b} \pm 0.2$	$6.7^{a} \pm 0.1$	$6.2^{bc} \pm 0.1$	$6.0^{c} \pm 0.4$	$6.0^{c} \pm 0.4$	$5.4^{d} \pm 0.1$
Total amino acids	$81.0^{d} \pm 2.0$	$86.4^{c} \pm 1.0$	$88.9^{bc} \pm 0.8$	$96.2^{a} \pm 2.6$	$91.6^{b} \pm 3.3$	$86.6^{c} \pm 2.1$

with MBDs 1, 2, and 3, which were made with decapsulated cysts of *Artemia* as total or partial protein source, achieved lower growth (58.9, 50.05, and 48.4 mg, respectively) than nauplii. However, their growth was significantly higher than the fish larvae fed MBD 4 (20.5 mg), which was made with fishmeal as sole protein source, and also higher than the larvae fed the commercial diet (24.8 mg). In general, the MBDs made with decapsulated cysts of *Artemia* were well accepted by the catfish larvae. The transparent gastrointestinal tract of the larvae allowed visual verification of gut fullness after every feeding. All the food particles were ingested 30 min after each meal. With regard to MBD 4 and the commercial diet, the fish ingested most of the particles.

Diet Proximate Composition

The protein, lipid and ash contents of the MBDs and *Artemia* instar I nauplii are presented in Table 1. More protein was contained in the nauplii, in the MBDs with fishmeal as protein source, and in the commercial diet compared to the other MBDs. The amino acid composition had small variations among the diets (Table 2). No significant differences ($P > 0.05$) were found for cystine and histidine. MBD 4 and the commercial diet contained more total amino acids. However, a higher total amino acid content did not yield higher fish growth. The lipid content was higher in MBD 3 and MBD 4 than in the other diets due to the inclusion of fish oil and fishmeal in their formulation (Table 1). Similarly, the levels of individual fatty acids differed significantly ($P < 0.05$) between diets (Table 3). In general, MBD 3 and 4 had significantly higher content of most fatty acids with exception of the C-18 group. C-18 fatty acids, especially linolenic acid (18:3n-3) was substantially higher in the diets containing *Artemia* cysts and in the nauplii than in the other diets. In MBD 1, 2, and 3, and in nauplii, the 18:3n-3 represented more than

TABLE 3. *Fatty acid composition (mg/g dw) of experimental microbound diets made with decapsulated cysts of* Artemia *(MBD 1 and MBD 2), a combination of cysts and fishmeal (MBD 3), and fishmeal (MBD 4) as protein sources, a commercial diet, and instar I nauplii of* Artemia. *Means in the same row with the same superscript are not significantly different (P > 0.05). Means and standard deviation values were rounded to decimals. Means were obtained with three replicates.*

	Diet					
Fatty acid	MBD 1	MBD 2	MBD 3	MBD 4	Com.	Nauplii
14:0	$1.4^{d} \pm 0.2$	$1.4^{d} \pm 0.0$	$6.3^{c} \pm 0.2$	$10.9^{a} \pm 0.1$	$10.2^{b} \pm 0.4$	$1.4^{d} \pm 0.1$
16:0	$13.8^{e} \pm 1.0$	$14.9^{d} \pm 0.1$	$21.9^{c} \pm 0.8$	$28.3^{a} \pm 0.1$	$23.7^{b} \pm 0.6$	$15.5^{d} \pm 0.2$
16:1n-7	$4.4^{e} \pm 0.4$	$4.7^{de} \pm 0.1$	$7.7^{b} \pm 0.3$	$10.6^{a} \pm 0.1$	$6.0^{c} \pm 0.2$	$5.0^{d} \pm 0.1$
18:0	$5.4^{c} \pm 0.3$	$6.1^{b} \pm 0.1$	$5.0^{d} \pm 0.2$	$4.2^{e} \pm 0.1$	$2.9^{f} \pm 0.2$	$6.6^{a} \pm 0.1$
18:1n-9	$21.5^{b} \pm 1.3$	$24.4^{a} \pm 0.3$	$18.2^{c} \pm 0.7$	$12.6^{d} \pm 0.1$	$11.8^{d} \pm 0.2$	$25.1^{a} \pm 0.2$
18:1n-7	$7.4^{c} \pm 0.5$	$8.4^{b} \pm 0.1$	$5.8^{d} \pm 0.2$	$3.1^{e} \pm 0.2$	$5.8^{d} \pm 0.4$	$9.8^{a} \pm 0.1$
18:2n-6-t	$0.2^{b} \pm 0.1$	$0.3^{a} \pm 0.0$	$0.2^{b} \pm 0.0$	$0.0^{d} \pm 0.0$	$0.1^{c} \pm 0.0$	$0.3^{a} \pm 0.0$
18:2n-6-c	$6.3^{b} \pm 0.4$	$7.0^{a} \pm 0.1$	$5.4^{c} \pm 0.2$	3.7e ± 0.0	$4.6^{d} \pm 0.5$	$7.4^{a} \pm 0.1$
18:3n-3	$34.1^{c} \pm 2.0$	$37.3^{b} \pm 0.3$	$20.4^{d} \pm 0.9$	$2.7^{e} \pm 0.2$	$2.0^{e} \pm 0.4$	$41.2^{a} \pm 0.6$
18:4n-3	$5.7^{c} \pm 0.4$	$6.1^{b} \pm 0.0$	$6.5^{a} \pm 0.3$	$6.8^{a} \pm 0.0$	$2.3^{d} \pm 0.1$	$6.5^{a} \pm 0.1$
20:1n-9	$0.4^{e} \pm 0.1$	$0.5^{de} \pm 0.0$	$4.7^{b} \pm 0.1$	$9.3^{a} \pm 0.0$	$1.2^{c} \pm 0.1$	$0.6^{d} \pm 0.0$
20:4n-6	$1.5^{c} \pm 0.1$	$1.7^{b} \pm 0.1$	$1.3^{d} \pm 0.1$	$0.8^{e} \pm 0.0$	$0.6^{f} \pm 0.0$	$2.0^{a} \pm 0.0$
20:4n-3	$0.9^{b} \pm 0.1$	$1.0^{a} \pm 0.1$	$1.1^{a} \pm 0.1$	$1.1^{a} \pm 0.0$	$0.3^{c} \pm 0.1$	$1.1^{a} \pm 0.0$
20:5n-3	$5.2^{e} \pm 0.2$	$5.7^{d} \pm 0.1$	$13.2^{b} \pm 0.4$	$21.1^{a} \pm 0.0$	$12.8^{b} \pm 0.3$	$6.8^{c} \pm 0.1$
21:5n-3	$0.1^{e} \pm 0.0$	trace	$0.4^{b} \pm 0.0$	$0.8^{a} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.2^{d} \pm 0.0$
22:6n-3	$0.2^{d} \pm 0.1$	$0.2^{d} \pm 0.0$	$11.6^{c} \pm 0.4$	$23.7^{a} \pm 0.2$	$12.4^{b} \pm 0.3$	$0.3^{d} \pm 0.0$
Σ n-3 $\geq$ 20:3n-3	7.3	8.0	27.6	48.4	26.9	9.4
Σ n-6 $\geq$ 18:2n-6-t	8.9	9.9	7.5	5.1	5.6	10.6
Total FAME	$123.6^{d} \pm 6.4$	$136.2^{c} \pm 0.8$	$151.2^{b} \pm 5.4$	$166.8^{a} \pm 0.4$	$106.6^{e} \pm 2.6$	$147.3^{b} \pm 1.5$

20% of the total fatty acid methyl ester (FAME) (Table 3). EPA (20:5n-3) and DHA (22:6n-3) were significantly higher in MBD 3 and 4, and in the commercial diet.

Diet Solubility and Protein Digestibility

The diet solubility was significantly different among the diets (Fig. 2a). The commercial diet was significantly less soluble than the MBDs. However, in the case of the commercial diet, a high percentage of insoluble matter was inversely related to the diet digestibility as shown by its lower *in vitro* protein digestibility (Fig. 2b). Although the solubility was higher in all MBDs compared to the commercial diet, leaching of protein into the water column was limited. The protein contents in the insoluble part after the solubility test for each diet were (in g/100 g dw): MBD 1 = 64.7; MBD 2 = 66.2; MBD 3 = 65.7; MBD 4 = 69.8; Com. = 64.2. These protein values are higher than the original ones before the diet solubility test (Table 1). This indicates that there is a reduced leaching of protein and that other diet components were dissolved in the water. The *in vitro* protein digestibility was significantly different ($P < 0.05$) among the diets (Fig. 2b). Higher protein digestibility was obtained with MBD 1 (86%) and the nauplii (78%), and the lowest protein digestibility was observed for the commercial diet (44%). MBD 2, 3, and 4 presented intermediate digestibility values between the highest and the lowest, with 59, 66, and 53%, respectively.

Protein Molecular Weight

The protein in the artificial diets, as analyzed by SDS-PAGE, showed different patterns between the *Artemia* cyst-based MBDs, the MBDs made with fishmeal, the commercial diet and *Artemia* nauplii (Fig. 3). The relative amounts of the different

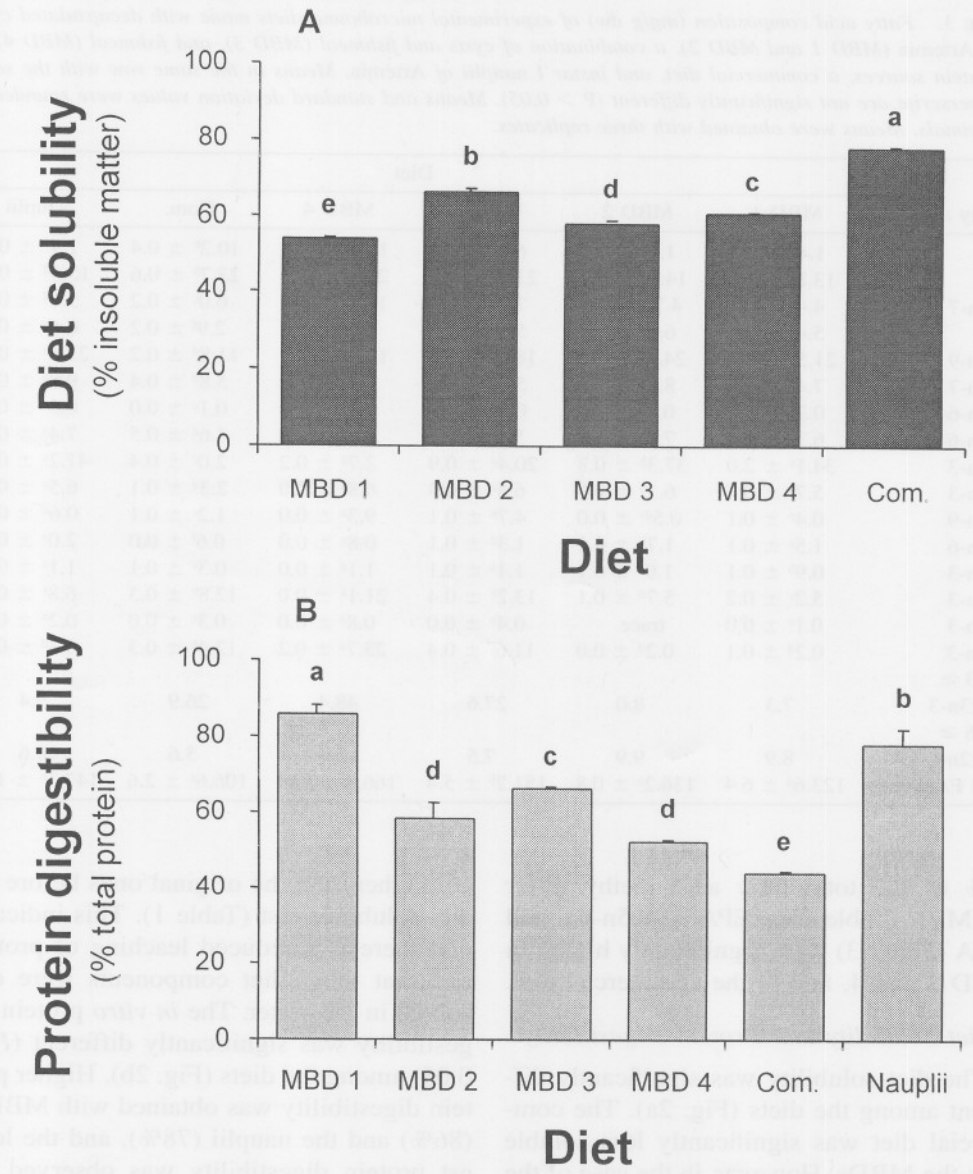

FIGURE 2. *Diet solubility (A) and protein digestibility* in vitro *(B) of four experimental microbound diets (MBD) and a commercial diet. The protein digestibility of instar I nauplii of* Artemia *was also determined. Bars with different letter are significantly different* ($P < 0.05$). *Means for diet solubility were determined with three replicates and for protein digestibility with two replicates.*

size fractions in the dietary protein are presented in Table 4. In the MBDs made with *Artemia* cysts, most of the protein had a molecular weight between 29.4 and 82 kDa. The fishmeal-based MBDs, the commercial diet, and the *Artemia* nauplii contained mainly smaller size proteins between 7.4 and 49.2 kDa. In the case of the nauplii, almost half of the protein consisted of low molecular weight proteins between 7.4 and 20.9 kDa. Heating of cysts at 80 C prior to diet preparation apparently increased the low molecular weight protein fraction of MBD 2 compared to the MBDs made with

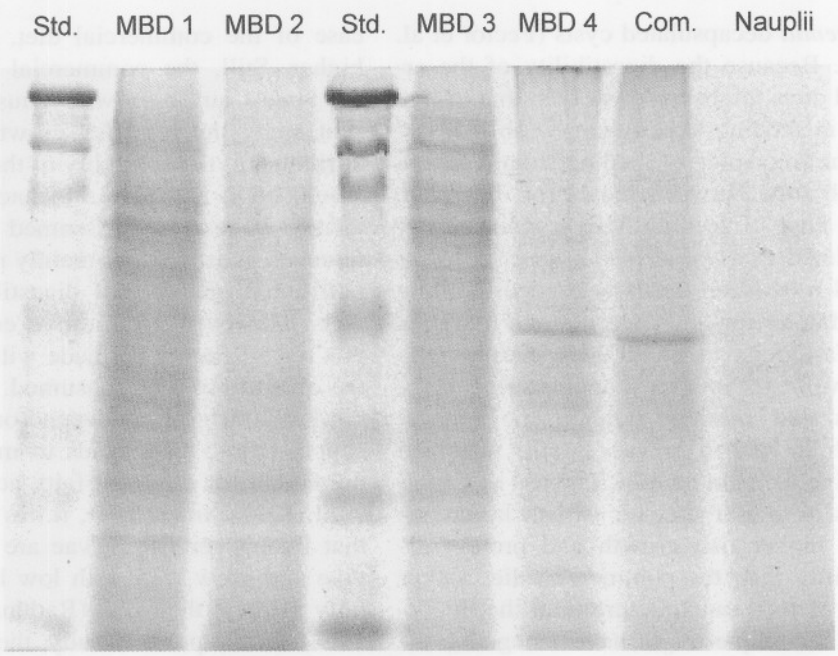

FIGURE 3. *SDS-PAGE analysis of the protein pattern of four experimental microbound diets made with* Artemia *decapsulated cysts (MBD 1 and MBD 2), a combination of cyst and fishmeal (MBD 3) and fishmeal (MBD 4) as protein sources, one commercial diet and instar I nauplii of* Artemia. *Std. = standard.*

non-heated decapsulated cysts. However, in both diets the profile of the different protein fractions was similar (Table 4). In MBD 3 containing 48% cysts and 52% fishmeal protein, the molecular weight profile of the proteins was more related to the profile of the cyst-based MBDs than to the fishmeal MBDs. The similarity of the protein bands in the gel (Fig. 3) and the protein distribution (Table 4) between the MBD 4 and the commercial diet suggest a strong similarity in the protein source used in the formulation of both diets.

Discussion

The growth of *C. gariepinus* larvae in the present study was higher than that obtained in previous feeding tests with MBDs made

TABLE 4. *Molecular weight (MW) of protein fractions extracted from microbound diets made of decapsulated cysts of* Artemia *(MBD 1 and MBD 2), a combination of cysts and fishmeal (MBD 3), and fishmeal (MBD 4) as protein sources, a commercial starter diet, and Instar I nauplii of* Artemia. *The results are expressed as percentage of the total protein in the gel for each diet.*

MW of protein standards[a] (kDa)	Diet					
	MBD 1	MBD 2	MBD 3	MBD 4	Com.	Nauplii
113.0–200.0	3.7	1.3	13.9	0	0	0
82.0–113.0	14.4	8.9	22.4	4.6	15.8	0
49.2–89.0	28.2	15.9	30.7	30.3	27.7	10.6
34.8–49.2	31.3	28.2	24.1	20.7	21.9	9.6
29.4–34.8	3.8	36.7	6.1	3.3	5.6	7.8
20.9–29.4	11.4	9.1	1.4	2.8	1.7	23.6
7.4–20.9	7.3	0	1.5	38.3	27.4	48.7

[a] SDS-PAGE standards, broad range 161–0318, Bio-Rad Laboratories, California, USA.

of *Artemia* decapsulated cysts (Pector et al. 1994). Because the digestibility of the artificial diets might be lower than that of live food, in feeding experiments with fish larvae, the frequency of feeding should ensure enough time between meals for digestion. In the case of feeding MBDs made of decapsulated cysts of *Artemia,* a period of at least 4 h between meals is needed to allow a good digestion and absorption of nutrients (García-Ortega et al. 2000a). Despite the improvements in diet manufacturing techniques and nutrient quality, the highest growth in catfish larvae is still achieved with live *Artemia* nauplii. The use of CMC-MBDs as starter diet for catfish larvae allowed higher fish growth and protein digestibility than the commercial diet. Other forms of feed manufacture than the microbound technique might have a negative effect on the protein quality due to severe conditions in which the feed components are processed (e.g., high temperatures and cooking pressures). CMC proved to be a good binder in the preparation of microbound diets that include decapsulated cysts of *Artemia* in their formulation. It allowed for a good solubility and high digestibility of the diet. Other binders (e.g., carrageenan) could reduce the digestibility of the protein and consequently limit their suitability as feed for fish larvae (Gawlicka et al. 1996). In the present study it was assumed that the fish acquired most of the protein in the diets. MBDs made with decapsulated cysts of *Artemia* were well accepted by catfish larvae and yielded higher growth and survival than fishmeal-based diets. The particle size was suitable for their ingestion and the insoluble matter was high enough to ensure that the fish larvae ingested all the protein contained in the diets. In a previous study with African catfish larvae (Pector et al. 1994) it was concluded that the differences in growth among fish fed MBDs were related to the differences in insoluble matter of the diets. However, in the present study the insoluble matter of the diets was similar among the experimental MBDs, and in the case of the commercial diet, it was even higher. Still, the commercial diet yielded the lowest larval growth. Thus, in the present study the low fish growth cannot be attributed to the solubility of the diet, which would be in any case a technological deficiency. Instead, it is assumed that the differences in growth are mainly related to the nutritional quality and digestibility of the diets. However, a definitive conclusion in this regard cannot be made without data on the quantity of diet consumed.

Marine fish larvae depend on the dietary supply of n-3 fatty acids to meet their requirement for essential fatty acids (Sargent et al. 1997). In contrast, it has been shown that freshwater fish larvae are able to survive and grow well with low levels of n-3 fatty acids in their diet (Radünz-Neto et al. 1994). In the present study, the nauplii and cysts of *Artemia* used were low in n-3 long-chain fatty acids (e.g., EPA and DHA). However, they contained a relatively high amount of linolenic acid (18:3n-3), which can be converted to EPA and DHA by freshwater fish larvae (Kanazawa 1985). In MBD 4, the supply of essential fatty acids was covered by a high supply of EPA and DHA from the fish oil and fishmeal. Therefore, the differences in growth of catfish larvae cannot be attributed to a deficiency of essential fatty acids in the diets. In the *Artemia*-based MBDs, the differences in protein content and total amino acids between MBD 1 and MBD 2 might be related to the strong heating of the cysts that was used in the latter. Heating at 80 C has detrimental effects on the protein quality of *Artemia* cysts (García-Ortega et al. 2000b). Strong heating might also reduce the amount of non-protein nutrients in the cysts, resulting in elevated levels of protein and total amino acids in MBD 2 when compared to MBD 1 (Tables 1, 2). In MBD 4 and the commercial diet, high amino acid contents did not yield higher fish growth. Similarly, the higher amount of protein with low molecular weight in those two diets did not ensure higher fish growth. This might

be related to the quality of the protein in the diets. In the case of *Artemia* nauplii, which showed a high amount of low molecular weight proteins, the fish growth was the highest. This can be partially explained by the effect of visual and chemical stimuli from *Artemia* to the fish (Kolkovski et al. 1997).

The structure of the protein molecules might have also affected the nutritional quality of *Artemia* cysts and nauplii. Although both have a similar nutritional composition, the nauplii might have a protein structure that differs from that of the cysts. *Artemia* nauplii have a high water content, and this might have an effect on the molecular arrangement of the protein. The sampled protein from nauplii was composed of small peptides of molecular weight smaller than 89 kDa, with most of it under 29.4 kDa. In the case of the cysts-based MBDs, the majority of the protein had a higher molecular weight between 34.8 to 113 kDa in MBDs 1 and 3, and 29.4 to 89 in MBD 2. Fish larvae grew equally well on those three diets. Heating the cysts at 80 C reduced the *in vitro* protein digestibility of MBD 2 (from 86% in MBD 1 down to 59% in MBD 2). Yet, fish larvae receiving MBD 2 grew better than the larvae receiving fishmeal-based MBD. The growth results with *Artemia* nauplii are consistent with the hypothesis that the use of low molecular weight proteins or free amino acids in artificial diets improves their utilization by the fish larvae (Fyhn 1989; Walford and Lam 1993). Moreover, it was also suggested that the incorporation of small peptides or protein hydrolysates in the diets improved the developmental processes in the digestive tract of fish larvae (Cahu and Zambonino Infante 1995; Cahu et al. 1999).

Decapsulated cysts improved the acceptability and digestibility of a dry artificial diet. In the present study *Artemia* cysts were used in combination with fishmeal (MBD 3). With this diet, larval growth was improved compared to the treatments where fishmeal was used as the sole protein source. Apparently the incorporation of 50% protein from cysts in the diet is enough to achieve this positive effect. The explanation for this positive effect has yet to be elucidated, but some factors can be mentioned. The cysts of *Artemia* might provide substances that work as chemical attractants, as occurs when live nauplii are fed to fish larvae (Kolkovski et al. 1997). Another explanation might be related to the effect of food processing on the protein-lipid complexes in the diet. Chobert and Haertlé (1997) stated that, while protein-lipid interactions are responsible for the functionality of the proteins, the structural complexes differ between those found in biological systems and those formed during food processing. Moreover, Rudloff and Lonnerdal (1992) demonstrated that the digestibility of dietary protein is reduced by the protein-lipid interactions after food processing. This is an aspect to consider further in the development of artificial diets for fish larvae. The *Artemia* embryos contained in the decapsulated cysts used represent a biological system in contrast to the fishmeal, which was used as an alternative protein ingredient. This difference might have affected the results. Thus, when developing artificial diets for fish larvae, the interactions between nutrients should be studied in parallel to dietary protein quality.

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